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# TruSight™ Tumor Sample Preparation Guide

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# Overview

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# Introduction

TruSight Tumor Sample Preparation takes a deeper view of variation in solid tumors including lung, colon, melanoma, gastric, and ovarian. This enables clinical researchers to look beyond point mutations within hot spots in single genes for a more comprehensive view of somatic variation. The TruSight Tumor content set provides amplicon-based library preparation reagents, DNA QC, sample indexes, and oligos targeting identified regions of interest. Sufficient reagents are supplied for 48 samples and indexes provided enable sample indexing of 4 samples per sequencing run. TruSight Tumor leverages the paired end read capability, speed, and high data quality of the MiSeq System providing on-instrument variant-calling software and cloud-based annotation and filtering software.

## High Accuracy, Low-Frequency Variant Detection

- Highly accurate somatic variant analysis at limit of detection below 5% allele frequency across 175 amplicons with 1000x minimum coverage of each region Optimized for formalin-fixed, paraffin-embedded (FFPE) tissues.

## Optimized for Formalin-Fixed, Paraffin-Embedded (FFPE) Tissues

- Exceptional sample success rate with minimal DNA input for accurate base calling even in degraded FFPE samples.

## Deep Coverage of Variants Involved with Solid Tumors

- Coverage of exon coding regions for analysis of molecular heterogeneity in highly relevant content selected from CAP and NCCN guidelines and late stage clinical trials.

# DNA Input Recommendations



Formalin-fixed, paraffin-embedded (FFPE) human tissues are a valuable source of material for molecular analysis and clinical studies. A number of processes and protocols now exist for the extraction and purification of nucleic acids from FFPE samples; however, as the assays used to evaluate DNA and RNA have evolved from simple monoplex PCR to higher plexity products, the quality and amount of nucleic acid extracted from FFPE material has become more critical to the success of these assays.

The TruSight Tumor Sample Preparation assay can be used to generate sequencing libraries that are highly multiplexed at both the target and sample level. The high level of assay complexity is enabled by combining an oligo extension/ligation process with universal PCR. Both of these reactions require a largely intact DNA template that can be denatured. The formalin fixation and paraffin embedding of tissues impacts this requirement by fragmenting, cross-linking, and otherwise damaging DNA through a variety of chemical modifications. Assessing the extent of this damage and adjusting the DNA extraction procedure to compensate for it as much as possible is essential to improving the success of the TruSight Tumor Sample Preparation assay with FFPE DNA.

## DNA Extraction Recommendations

Illumina recommends the *Qiagen Supplementary Protocol: Purification of genomic DNA from FFPE tissue using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution* for the purposes of extracting the highest amount of amplifiable DNA from an FFPE tissue block, with the following modifications:

- Extract gDNA from 8 separate 5 µM FFPE tissue sections.
- Deparaffinize with 320 µl of Qiagen Deparaffinization Solution.
- Digest with proteinase K in a thermomixer overnight at 1,000 rpm.
- Decrease elution volume to 30 µl to maximize DNA concentration.

# Additional Resources

The following resources are available for TruSight Tumor Sample Preparation protocol guidance and sample tracking. Access these and other resources on the Illumina website at support.illumina.com/sequencing/kits.ilmn. Then, select **TruSight Tumor Sample Preparation Support**.

| Resource | Description |
|---|---|
| Best Practices | Provides best practices specific to this protocol. Review this before starting sample preparation. Topics include:<br>• General Advice on Sample Handling<br>• Ensuring Consistency<br>• Handling Magnetic Beads<br>• Handling Reagents<br>• Avoiding Cross-Contamination<br>• Washing with 80% Ethanol During PCR Clean-Up<br>Click **Best Practices** on the **TruSight Tumor Sample Preparation Support** page. |
| TruSight Tumor Sample Preparation Experienced User Card (part # 15038313) | Provides protocol instructions, but with less detail than what is provided in this user guide. **New or less experienced users are strongly advised to follow this user guide and not the EUC.**<br>Click **Documentation & Literature** on the **TruSight Tumor Sample Preparation Support** page. |
| Illumina Experiment Manager (IEM) | Enables you to create and edit appropriate sample sheets for Illumina sequencers and analysis software and record parameters for your sample plate.<br>To download the software, click **Downloads** on the **TruSight Tumor Sample Preparation Support** page.<br>To download the documentation, click **Documentation & Literature** on the **TruSight Tumor Sample Preparation Support** page. |

# TruSight Tumor Protocol

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# Introduction

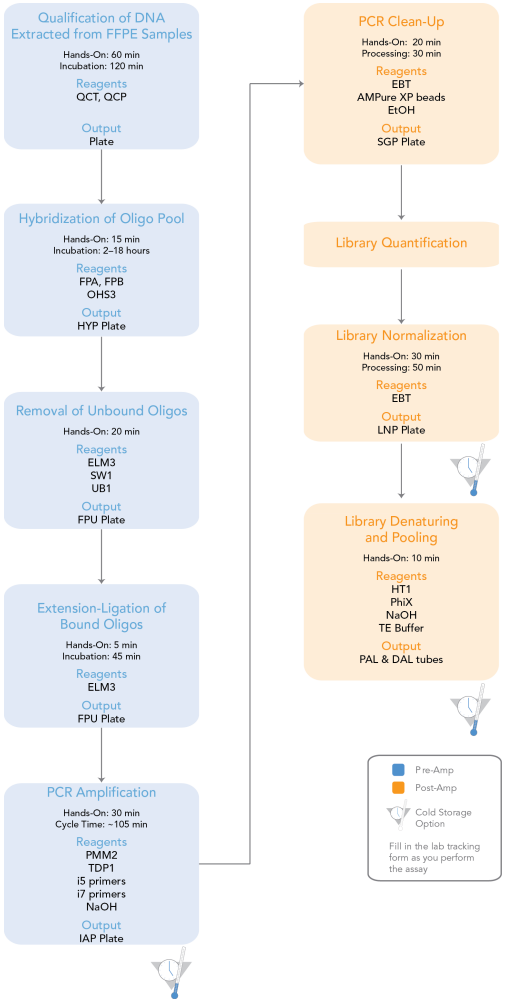
This chapter describes the TruSight Tumor protocol.

- Review Best Practices before proceeding. See *Additional Resources* on page 4 for information on how to access TruSight Tumor Best Practices on the Illumina website.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- If you are pooling, record information about your samples before beginning library preparation for later use in data analysis.
  - Use IEM to create and edit well formed sample sheets for Illumina sequencers and analysis software. See *Additional Resources* on page 4 for information on how to download IEM software and documentation from the Illumina website.
- As a troubleshooting aid, ACD1 (Amplicon Control DNA), and ACP1 (Amplicon Control Oligo Pool) have been included in this kit. Using ACD1 instead of gDNA and ACP1 instead of FPA and FPB in the TruSight Tumor assay can help narrow down issues arising from gDNA sample prep or primer contamination. Libraries made with these controls cannot be sequenced along side other TruSight Tumor libraries as they require longer cycles.

# TruSight Tumor Sample Preparation Workflow

The following diagram illustrates the workflow using the TruSight Tumor Sample Preparation Kit. Safe stopping points are marked between steps.

Figure 1 TruSight Tumor Sample Preparation Workflow



# Qualification of DNA Extracted from FFPE Samples

During this step, a qPCR reaction will be performed to determine the amplifiability of your FFPE-extracted gDNA samples. By comparing the amplifiability of FFPE DNA relative to that of the QCT non-FFPE reference gDNA, a ΔCq value can be calculated for each sample and used to predict its performance in the TruSight Tumor Sample Preparation assay. The exact amount of FFPE DNA input will vary according to the quality of the extracted DNA.

## Estimated Time

- Total duration: 3 hours
- Hands-on: 60 minutes

## Consumables

| Item | Quantity | Storage | Supplied By |
|---|---|---|---|
| QCT (Quality Control Template) | 1 tube | -15° to -25°C | Illumina |
| QCP (Quality Control Primer) | 1 tube | -15° to -25°C | Illumina |
| Genomic DNA<br>See *DNA Input Recommendations* on page 3. | As needed | -15° to -25°C | User |
| KAPA SYBR FAST Master Mix Universal (2x)<br>(Other qPCR mixes can be used but should be tested.) | 1 plate | -15° to -25°C | User |
| Nuclease-free water | As needed | | User |
| 48 or 96-well plate | 1 plate | | User |

# Preparation

1 Remove the QCT, QCP, KAPA SYBR FAST Master Mix Universal (2x), and genomic DNA from -15° to -25°C storage and thaw at room temperature for up to 30 minutes.

2 Place thawed tubes on ice.

3 If using QCT for the first time, aliquot 5 µl of QCT into different PCR strip tubes for long-term storage to avoid freeze-thawing.

## Procedure

1 Add 5 µl of QCT to 495 µl of Nuclease-free water in a microfuge tube.

2 Vortex the dilution to thoroughly mix the sample.

3 Add 1 µl of QCP to 9 µl of Nuclease-free water in a microfuge tube.

NOTE
Make a larger dilution if qualifying more than one genomic DNA sample.

4 Vortex the dilution to thoroughly mix the sample.

5 Add 1.5 µl of Qiagen-extracted genomic DNA to 148.5 µl of Nuclease-free water in microfuge tubes to make a 100-fold dilution.

6 Vortex the dilutions to thoroughly mix the samples.

7 Determine the plate layout of the qPCR reaction. For 10 samples, Illumina recommends this plate layout:

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | QCT | Sample 1 | Sample 3 | Sample 5 | Sample 7 | Sample 9 |
| B | QCT | Sample 1 | Sample 3 | Sample 5 | Sample 7 | Sample 9 |
| C | QCT | Sample 1 | Sample 3 | Sample 5 | Sample 7 | Sample 9 |
| D | NTC* | Sample 2 | Sample 4 | Sample 6 | Sample 8 | Sample 10 |
| E | NTC* | Sample 2 | Sample 4 | Sample 6 | Sample 8 | Sample 10 |
| F | NTC* | Sample 2 | Sample 4 | Sample 6 | Sample 8 | Sample 10 |

NTC: No template control. Illumina recommends using nuclease-free water.

8 Prepare the SYBR master mix reaction as follows. The master mix contains extra volume:

Table 1 SYBR Master Mix Reactions

| Consumable | µl per well | µl per plate (48-wells) | µl per plate (96-wells) |
|---|---|---|---|
| KAPA SYBR FAST Master Mix Universal (2x) | 5.0 µl | 275 µl | 550 µl |
| QCP | 1.0 µl | 55 µl | 110 µl |
| Nuclease-free water | 2.0 µl | 110 µl | 220 µl |

9 Mix gently but thoroughly.

10 Place the reaction mix on ice and protect it from light until use.

11 Add 8 µl of the master mix to each well of the plate. Take care to pipette accurately into the wells as small variations will affect the assay.

12 Add 2 µl of the QCT dilution, the sample dilutions, or nuclease-free water to each well of the plate. Take care to pipette accurately into the wells as small variations will affect the assay.

13 Seal the plate with the appropriate seal (depending on the instrument that you are using), taking care to avoid cross-contamination and to avoid smudging the surface of the lids.

14 Place the plate on an adapter (if needed) and centrifuge the plate to 250 xg for 1 minute.

15 Ensure the seal is free of any liquid or dust, place the plate on the qPCR machine in the correct orientation, then close the lid and run the following thermal profile:

| Procedure | Temperature | Time |
|---|---|---|
| Hot Start | 50°C | 2 minutes |
| | 95°C | 10 minutes |
| x40 | 95°C | 30 seconds |
| | 60°C | 30 seconds |
| | 72°C | 30 seconds |

16 Confirm that the instrument captures images after the 72°C step.

NOTE
The Cq threshold should be set to a value that avoids inaccurate measurements due to background (100 RFU on the BioRad 396CFX System).

17 After the final step, the thermal cycler analyzes the quantified libraries. Make sure that amplification of the NTC occurs at least 10 cycles after QCT amplification.

18 Ensure that there is good amplification for the QCT and remove outliers from a triplicate group that are > 0.5 Cq different from the rest of the group.

NOTE
Four or more outliers per plate indicate technical errors.

19 Replicates exhibiting abnormal amplification curves should be excluded (see Illumina sequencing white paper, *Generating Sequencing Libraries Using DNA from FFPE Samples* for more details).

20 Subtract the average Cq for the QCT from the average Cq for each sample to yield the ΔCq values for each sample.

# Hybridization of Oligo Pool

During this step, a custom pool containing upstream and downstream oligos specific to your targeted regions of interest is hybridized to your genomic DNA samples.

WARNING

**This set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.**

For more information, see the MSDS for this kit, at http://www.illumina.com/msds.

NOTE

Illumina does not support the use of gDNA samples giving a delta Cq value of greater than 4.

## Estimated Time

- Total duration: 14–18 hours (overnight)
- Hands-on: 15 minutes

## Consumables

| Item | Quantity | Storage | Supplied By |
|---|---|---|---|
| TruSight Tumor Sample Preparation Oligo Pools (FPA, FPB) | 1 tube per pool | -15° to -25°C | Illumina |
| OHS3 (Oligo Hybridization for Sequencing 3) | 1 tube | -15° to -25°C | Illumina |
| Genomic DNA<br>See *DNA Input Recommendations* on page 3. | As needed | -15° to -25°C | User |
| 96-well skirted PCR plate | 1 plate | | User |
| [Optional] Adhesive aluminum foil seal if a heat sealer is not available | 2 seals | | User |
| Troughs | As needed | | User |

## Preparation

1. Remove the TruSight Tumor Sample Preparation Oligo Pools (FPA, FPB) and genomic DNA from -15° to -25°C storage and thaw at room temperature for up to 30 minutes. Place thawed tubes on ice. If running Illumina-provided controls, thaw ACD1 and/or ACP1 as well.
2. Set a 96-well heat block to 95°C.
3. Remove OHS3 from -15° to -25°C storage and thaw at room temperature. If precipitate is observed, incubate at 37°C for 10 minutes and vortex 1 minute. Repeat as needed until precipitate is no longer visible.

NOTE
Using the provided controls enables Illumina Technical Support to troubleshoot in the event you need assistance. Illumina technical support recommends including control samples in your assay periodically to establish baselines and monitor overall performance.

NOTE
The control ACP1 is specific for Homo sapiens and will not work with DNA from other species.

## Procedure

1. Label a new 96-well PCR plate "**HYP_Plate_ID**".
2. Dilute 10 µl of genomic DNA extracted from FFPE samples. Use the table below to determine the fold dilution required for each calculated delta Cq:

| Delta Cq | -2.5 to -1.5 | -1.5 to -0.5 | -0.5 to 0.5 | 0.5 to 1.5 | 1.5 to 4 |
|---|---|---|---|---|---|
| Dilution | 16x | 8x | 4x | 2x | No dilution |

NOTE
If preparing libraries from the control DNA in parallel with FFPE DNA samples, dilute 5 µl of ACD1 with 45 µl of TE Buffer. Add 10 µl to each of two control wells for FPA and FPB, and/or two control wells for ACP1.

3 Add 10 µl of each sample as prepared in step 2 to wells on the left half of the **HYP** plate, starting with column 1. Then, repeat this process on the right half of the **HYP** plate, starting with column 7.

4 Using a multichannel pipette, add 5 µl of oligo pool 1 (FPA) to all sample-containing wells on the left half of the **HYP** plate. Then, add 5 µl of oligo pool 2 (FPB) to all sample-containing wells on the right half of the **HYP** plate. Change tips after each column to avoid contamination.

NOTE
If preparing libraries from ACD1, add 5 µl of FPA to one control well and 5 µl of FPB to the second control well. If preparing libraries using ACP1, add 5 µl of ACP1 to two additional control wells of ACD1.

5 Using a multichannel pipette, add 35 µl of OHS3 to each sample in the **HYP** plate. Gently pipette up and down 3–5 times to mix. Change tips after each column to avoid contamination.

NOTE
Ensure any crystals or precipitate in OHS3 have dissolved after following procedure described in Preparation step 3.

6 Seal the **HYP** plate with a heat sealer (Illumina recommends the Agilent PlateLoc Thermal Microplate Sealer). If a heat sealer is not available use an aluminum foil seal. Centrifuge at 1,000 xg at 20°C for 1 minute.

7 Place the **HYP** plate in the pre-heated block at 95°C and incubate for 1 minute.

8 Change the temperature of the same heat block to 40°C, and incubate for 14–18 hours.

NOTE
Moving the plate from the 95°C heat block to another pre-heated block set to 40°C will adversely affect hybridization.

# Removal of Unbound Oligos

This process removes unbound oligos from genomic DNA using a filter capable of size selection. Two wash steps using SW1 ensure complete removal of unbound oligos. A third wash step using UB1 removes residual SW1 and prepares samples for the extension-ligation step.

WARNING

**This set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.**

For more information, see the MSDS for this kit, at http://www.illumina.com/msds.

WARNING

This set of reagents contains β-mercaptoethanol. The following procedure may be performed in a hood or well-ventilated area if desired.

## Estimated Time

- Total duration: 20 minutes
- Hands-on: 20 minutes

## Consumables

| Item | Quantity | Storage | Supplied By |
|---|---|---|---|
| ELM3 (thawed in preparation for Extension-Ligation) | 1 tube | -15° to -25°C | Illumina |
| SW1 (Stringent Wash 1) | 1 tube | 2° to 8°C | Illumina |
| UB1 (Universal Buffer 1) | 1 tube | 2° to 8°C | Illumina |
| Filter plate with lid | 1 plate | | Illumina |
| Adapter collar (reusable) | 1 plate | | Illumina |
| MIDI plate | 1 plate | | User |
| Troughs | As needed | | User |

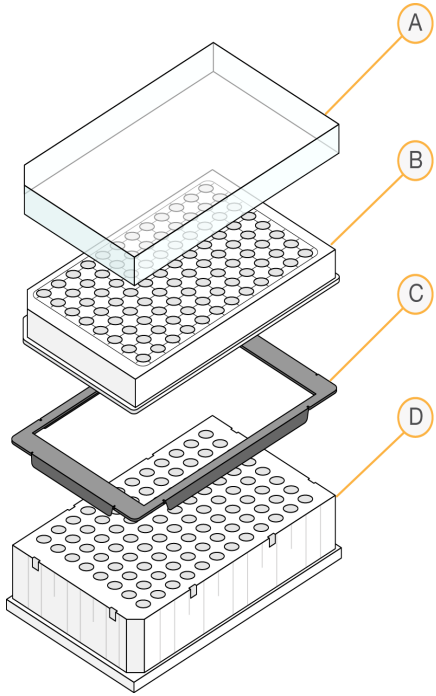
## Preparation

1. Remove ELM3 from -15° to -25°C storage and thaw at room temperature.
   ELM3 is used in the Extension-Ligation step and takes approximately 20 minutes to thaw.
2. Remove SW1 and UB1 from 2° to 8°C storage and set aside at room temperature.

3 Assemble the filter plate assembly unit (**FPU**) in the following order (from top to bottom):

NOTE
For instructions on viewing a video demonstration of this step see page 8.

Figure 2 Filter Plate Unit Assembly




**A** Lid
**B** Filter plate
**C** Adapter collar
**D** MIDI plate

4 Apply the **FPU** barcode plate sticker to the filter plate.

5 Pre-wash the **FPU** plate membrane as follows:

a Using a multichannel pipette, add 50 µl of SW1 to each well.

NOTE
Pre-wash only the wells to be used in the current assay. You may use fresh/unused wells of a previously opened filter plate, but wells that have been used in a previous assay should never be re-used.

b Cover the **FPU** plate with the filter plate lid and keep it covered during each centrifugation step.

c Centrifuge the **FPU** at 2,400 xg at 20°C for 5 minutes.

6 Pre-heat incubator (not heat block) to 37°C.

NOTE
Illumina strongly recommends keeping spare filter plates (FC-130-1006) on hand as general lab supplies.

## Procedure

1 After the overnight incubation, confirm the heat block has cooled to 40˚C.

NOTE
If the heat block fails to cool to 40˚C overnight, library preparation will need to be repeated.

2 Remove the **HYP** plate from the heat block and centrifuge at 1,000 xg at 20°C for 1 minute to collect condensation.

3 Using a multichannel pipette set to 60 µl, transfer the entire volume of each sample onto the center of the corresponding pre-washed wells of the **FPU** plate. Change tips after each column to avoid cross-contamination.

4 Cover the **FPU** plate with the filter plate lid and centrifuge the **FPU** at 2,400 xg at 20°C for 5 minutes.

5 Wash the **FPU** plate as follows:

a Using a multichannel pipette, add 50 µl of SW1 to each sample well.

b Cover the **FPU** plate with the filter plate lid and centrifuge the **FPU** at 2,400 xg for 5 minutes.

6 Repeat the wash as described in the previous step.

7 Discard all the flow-through (containing formamide waste and unbound oligos) collected up to this point in an appropriate hazardous waste container, then reassemble the **FPU**. The same MIDI plate can be re-used for the rest of the pre-amplification process.

8 Using a multichannel pipette add 45 µl of UB1 to each sample well.

NOTE
Make sure that all liquid has drained after centrifugation. Repeat centrifugation if necessary. Any residual wash buffer may inhibit subsequent enzymatic reactions.

9 Cover the **FPU** plate with the filter plate lid and centrifuge the **FPU** at 2,400 xg for 5 minutes.

# Extension-Ligation of Bound Oligos

This process connects the hybridized upstream and downstream oligos. A DNA polymerase extends the upstream oligo through the targeted region, and is ligated to the 5′ end of the downstream oligo using a DNA ligase. This results in the formation of products containing the targeted regions of interest flanked by sequences required for amplification.

### Estimated Time

- Total duration: 50 minutes
- Hands-on: 5 minutes

### Consumables

| Item | Quantity | Storage | Supplied By |
|---|---|---|---|
| ELM3 (Extension-Ligation Mix 3) | 1 tube | -15° to -25°C | Illumina |
| Adhesive aluminum foil seal | 1 seal | | User |
| Troughs | As needed | | User |

## Procedure

1. Using a multichannel pipette, add 45 µl of ELM3 to each sample well of the **FPU** plate. The Extension-Ligation reaction takes place on the filter plate membrane. Changing tips between columns is not required if you use care to avoid cross-contamination.
2. Seal the **FPU** plate with adhesive aluminum foil, and then cover with the lid to secure the foil during incubation.
3. Incubate the entire **FPU** assembly in the pre-heated 37°C incubator for 45 minutes. Ensure waste is removed from bottom of the plate.
4. While the **FPU** plate is incubating, prepare the **IAP** (Indexed Amplification Plate) as described in the following section.

# PCR Amplification

In this step, the extension-ligation products are amplified using primers that add index sequences for sample multiplexing (i5 and i7) as well as common adapters required for cluster generation (P5 and P7).

## Estimated Time

- Total duration: ~100 minutes (depending on thermocycler)
- Hands-on: 30 minutes

## Consumables

| Item | Quantity | Storage | Supplied By |
|---|---|---|---|
| PMM2 (PCR Master Mix 2) | 1 tube | -15° to -25°C | Illumina |
| i5 primers (A5XX) | 1 tube per primer | -15° to -25°C | Illumina |
| i7 primers (A7XX) | 1 tube per primer | -15° to -25°C | Illumina |
| TDP1<br>(TruSeq DNA Polymerase 1) | 1 tube | -15° to -25°C | Illumina |
| Microseal 'B' adhesive film | 1 | | User |
| 0.05 N NaOH (freshly prepared from 10 N NaOH) | As needed | | User |
| 96-well skirted PCR plate | 1 plate | | User |
| Troughs | As needed | | User |

# Preparation

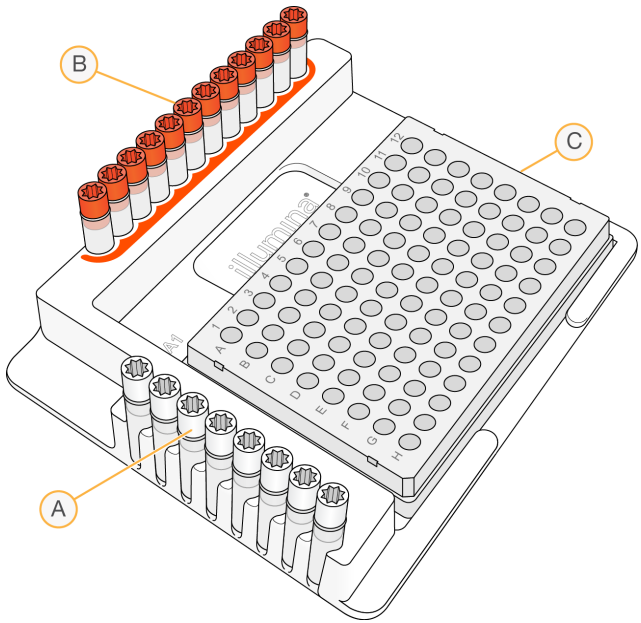
1. Prepare fresh 0.05 N NaOH by adding 20 µl of 10 N NaOH to 3.98 ml of sterile water.
2. Remove PMM2 and the index primers (i5 and i7) from -15° to -25°C storage and thaw on a bench at room temperature. Vortex each tube to mix and briefly centrifuge the tubes in a microcentrifuge.

3 Arrange i5 primer tubes (white caps, clear solution) vertically in the Index Plate Fixture, aligning tubes A501 through A508 with rows A through H.

4 Arrange i7 primer tubes (orange caps, yellow solution) horizontally in the Index Plate Fixture, aligning tubes A701 through A712 with columns 1 through 12. **Confirm i5 and i7 primer tubes are positioned correctly according to** Figure 3. Collect all liquid in the bottoms of the tubes by holding them in place in the rack and tapping it against the bench.

NOTE
If less than 48 samples (96 reactions) are being prepared or alternate index combinations are being employed, the Index Plate Fixture does not need to be used and the indices can be added to the appropriate wells of the IAP plate manually.

Figure 3 TruSeq Index Plate Fixture




A i5 primers (white caps)
B i7 primers (orange caps)
C **IAP** plate

5 Label a new 96-well PCR plate **IAP** (Indexed Amplification Plate).

6 Using a multichannel pipette, add 9 µl of i5 primers (clear solution) to each column of the **IAP** plate. ***Tips must be changed after each row to avoid index cross-contamination.***

7 To avoid index cross-contamination, discard the original *white* caps and apply new *white* caps.

8 Using a multi-channel pipette, add 9 µl of i7 primers (yellow solution) to each row of the **IAP** plate. ***Tips must be changed after each row to avoid index cross-contamination.***

9 To avoid index cross-contamination, discard the original *orange* caps and apply new *orange* caps.

10 For 96 reactions, add 60 µl of TDP1 to 2.58 ml of PMM2. If preparing fewer reactions, use the following calculation: # of reactions * (0.625 µl TDP + 26.875 µl PMM2). Do not pipette volumes of less than 5 µl of TDP1. Invert the PMM2/TDP1 PCR master mix 20 times to mix well. You will add this mix to the **IAP** plate in the next section.

NOTE
Do not vortex.

## Procedure

1 When the 45-minute extension-ligation reaction is complete, remove the **FPU** from the incubator. Remove the aluminum foil seal and replace with the filter plate lid. Removing the aluminum foil seal before centrifugation is recommended to ensure the reaction supernatant will drain into the waste plate effectively.

2 Centrifuge the **FPU** at 2,400 xg for 2 minutes.

3 Using a multichannel pipette, add 25 µl of 0.05 N NaOH to each sample well on the **FPU** plate. Ensure the NaOH is fully dispersed across each membrane by gently pipetting up and down if necessary.

4 Incubate the **FPU** plate at room temperature for 5 minutes.

5 While the **FPU** plate is incubating, use a multichannel pipette to transfer 22 µl of the PMM2/TDP1 PCR master mix to each well of the **IAP** plate containing index primers. Change tips between samples.

6 Transfer samples eluted from the **FPU** plate to the **IAP** plate as follows:

a Set a multichannel P20 pipette to 20 µl.

b Pipette the contents in the first column of the **FPU** plate up and down 5–6 times, then transfer 20 µl from the **FPU** plate to the corresponding column of the **IAP** plate. Gently pipette up and down 5–6 times to thoroughly combine the DNA with the PCR master mix.

NOTE
Slightly tilt the **FPU** plate to ensure complete aspiration and to avoid air bubbles.

c Transfer the remaining columns from the **FPU** plate to the **IAP** plate in a similar manner. Tips must be changed after each column to avoid index and sample cross-contamination.

d After all the samples have been transferred, the waste collection MIDI plate of the **FPU** can be discarded. The metal adapter collar should be put away for future use.

7 Cover the **IAP** plate with Microseal 'B' and seal with a rubber roller.

8 Centrifuge at 1,000 xg at room temperature for 1 minute.

9 Transfer the **IAP** plate to the post-amplification area.

10 Perform PCR on a thermal cycler using the following program:

- 95°C for 3 minutes
- 27 cycles of:
  - 95°C for 30 seconds
  - 62°C for 30 seconds
  - 72°C for 60 seconds
- 72°C for 5 minutes
- Hold at 10°C

NOTE
On the PCR machine, set the reaction volume to 60 µl and the temperature ramp speed to maximum.

SAFE STOPPING POINT
If you do not plan to immediately proceed to PCR Clean-Up following the completion of PCR, the plate can remain on the thermal cycler overnight, or store it at 2° to 8°C up to 48 hours.

# Verify Library Preparation (Optional)

After PCR, combine 5 µl of amplified product with 15 µl of DEPC/DI H20 and run on a 4% TBE agarose gel along with 100 bp ladder to confirm the presence of the 300–330 bp library product. If generating libraries with ACP1, the product should be present at 350–380 bp. Alternatively, the products can be run on a bioanalyzer.

# PCR Clean-Up

This process uses AMPure XP beads to purify the PCR products from the other reaction components.

### Estimated Time

- Total duration: 50 minutes
- Hands-on: 20 minutes

### Consumables

| Item | Quantity | Storage | Supplied By |
|---|---|---|---|
| EBT (Elution Buffer with Tris) | 1 tube | Room temperature | Illumina |
| AMPure XP beads | 440 µl per 8 samples | 2° to 8°C | User |
| 80% Ethanol, freshly-prepared | 3.3 ml per 8 samples | Room temperature | User |
| 96-well MIDI plates | 2 | | User |
| Microseal 'B' adhesive film | As needed | | User |
| Troughs | As needed | | User |

## Preparation

1 Bring the AMPure XP beads to room temperature.

2 Prepare fresh 80% ethanol from absolute ethanol.

NOTE
Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air impacting your results.

## Procedure

1 Centrifuge the **IAP** plate at 1,000 xg at 20°C for 1 minute to collect condensation.

2 Label a new MIDI plate **CLP_Plate_ID** (Clean-up Plate).

3 Invert AMPure XP beads 10 times. Vortex vigorously and then invert again 10 times.

NOTE
Immediately proceed to the next step to avoid settling of the beads.

4 Using a multichannel pipette, add 55 µl of AMPure XP beads to each well of the **CLP** plate.

5 Using a multichannel pipette set to 60 µl, transfer 55 µl PCR product from the **IAP** plate to the **CLP** plate. Change tips between samples.

6 Seal the **CLP** plate with Microseal 'B'.

7 Shake the **CLP** plate on a microplate shaker at 1,800 rpm for 2 minutes.

8 Incubate at room temperature without shaking for 10 minutes.

9 Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.

10 Using a multichannel pipette set to 100 µl and with the **CLP** plate on the magnetic stand, carefully remove and discard the supernatant. Change tips between samples.

NOTE
Delays during this step may lead to bead clumping following removal of the supernatant. Proceed immediately to next step as soon as all supernatant is removed.

11 With the **CLP** plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:

a Add 200 µl of freshly prepared 80% ethanol to each sample well using a multichannel pipette. Avoid disturbing the beads

b Incubate the plate on the magnetic stand for 30 seconds or until the supernatant appears clear.

c Carefully remove and discard the supernatant.

12 Repeat the 80% ethanol wash described in the previous step. Use a P20 multichannel pipette to remove excess ethanol. Aspirate all remaining ethanol using a fine tip pipette. Do not disturb or touch the beads.

13 Remove the **CLP** plate from the magnetic stand and allow the beads to air-dry for 5 minutes.

14 Using a multichannel pipette, add 40 µl of EBT to each sample.

Changing tips is not required if you use care to avoid cross-contamination.

15 Seal the **CLP** plate with Microseal 'B' and shake on a microplate shaker at 1,800 rpm for 5 minutes. After shaking, if any samples are not resuspended, gently pipette up and down or lightly tap the plate on the bench to mix, then repeat this step.

16 Incubate at room temperature without shaking for 2 minutes.

17 Place the plate on the magnetic stand for 2 minutes.

18 Label a new 96-well PCR plate **SGP** (Storage Plate).

19 Carefully transfer 40 µl of the supernatant from the **CLP** plate to the **SGP** plate. Change tips between samples.

20 Seal the **SGP** plate with Microseal 'B' and then centrifuge at 1,000 xg for 1 minute.

SAFE STOPPING POINT

After PCR-Clean Up, the **SGP** plate may be kept at room temperature during the upcoming library quantification and normalization steps. Once quantified samples have been normalized, the **SGP** plate may be stored at -20°C until ready to quantify and normalize any remaining samples.

# Library Quantification

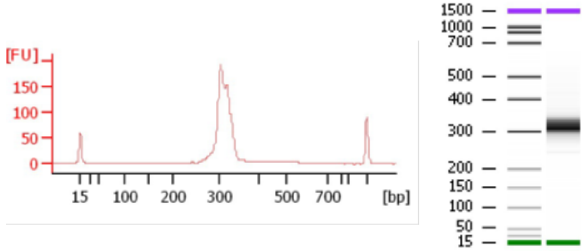
In order to achieve the highest quality of data on the Illumina MiSeq sequencing platform, it is important to create optimum cluster densities. This requires accurate quantitation of DNA libraries. Illumina recommends quantifying libraries generated from FFPE samples using the Agilent Technologies Bioanalyzer 2100.

## Quality Control

1 Load 1 µl of the re-suspended library on an Agilent Technologies 2100 Bioanalyzer using the Agilent DNA-1000. Refer to Agilent DNA Kit Guide (Part Number G2938-90014) and Agilent DNA 1000 Kit Quick Start Guide (Part Number G2938-90015) for complete instructions on using the Agilent Technologies 2100 Bioanalyzer.

2 Check the size and purity of the sample. The final product should be a band at ~300–330 bp as in Figure 4. If generating libraries using ACP1, the final product will be present at ~350–380 bp. This concentration should correlate with the relative library intensities previously observed on the agarose gel (see *Verify Library Preparation (Optional)* on page 25).

Figure 4 Representative DNA Sample Prep Library Size Distribution, 300–330 bp




NOTE

If >10% of the library product is present in the 150–250 bp range, we recommend repeating the *PCR Clean-Up* Procedure using 40 µl of product and 32 µl of AMPure XP beads.

# Library Normalization

This process normalizes the quantity of each library to ensure more equal library representation in your pooled sample.

## Estimated Time

- 30 minutes

## Consumables

| Item | Quantity | Storage | Supplied By |
|---|---|---|---|
| EBT (Elution Buffer with Tris) | As needed | Room temperature | Illumina |
| MIDI plate | As needed | | User |
| Microseal 'B' adhesive film | As needed | | User |

## Procedure

1. From the Agilent Bioanalyzer run, determine the concentration for all samples, add all concentrations (in terms of nM) for all peaks in the 300–330 bp range (or 350–380 bp for ACP1 libraries). The expected concentration range is 4–300 nM. Record the values.
2. Label a MIDI plate **LNP_plate** (Library Normalization Plate) and dilute 4 µl of all samples >20 nM to 4 nM with the EBT buffer (e.g. if a sample is 254 nM, add 4 µl of library to 250 µl of EBT buffer to give 4 nM).
3. For any samples ≤20 nM, dilute as needed to ensure that the volume of the final 4 nM working stock is at least 20 µl.

SAFE STOPPING POINT

If you do not plan to proceed to *Library Denaturing and Pooling on page 31* and subsequent sequencing on the MiSeq after completion of *Library Normalization*, store the sealed **LNP** plate at -15° to -25°C for up to 7 days.

# Library Denaturing and Pooling

In preparation for cluster generation and sequencing, equal volumes of normalized library are denatured, then are combined, diluted in hybridization buffer, and heat denatured prior to sequencing on the MiSeq. PhiX is used as an internal control for sequencing.

### Estimated Time

- Total duration: 10 minutes
- Hands-on: 10 minutes

### Required Equipment

- Heat block with microcentrifuge tube insert

### Consumables

| Item | Quantity | Storage | Supplied By |
|---|---|---|---|
| HT1 (Hybridization buffer) | 1 tube | -15° to -25°C | Illumina |
| 2 µl 10 nM PhiX Library | 2 µl | -15° to -25°C | Illumina |
| Stock 1.0 N NaOH (diluted to 0.1 N NaOH) | | 15° to 25°C | User |
| Laboratory-grade water | | | User |
| 1X TE Buffer | | | User |
| Microcentrifuge tubes (screwcap recommended) | 2 tubes | | User |
| PCR eight-tube strip | 1 | | User |
| 2.5 L Ice bucket | 1 | | User |

## Preparation

1 Set a heat block with a microcentrifuge tube insert to 96°C.

2 In an ice bucket, prepare an ice-bath. Place thawed HT1 in bath to chill.

3 Prepare a fresh dilution of 0.1 N NaOH by adding 100 µl stock 1 N NaOH to a microcentrifuge tube containing 900 µl laboratory-grade water.

CAUTION
Using freshly diluted NaOH is essential in order to completely denature samples for cluster generation on the MiSeq. Preparing a volume of 1 ml prevents small pipetting errors from affecting the final NaOH concentration.

4 Invert the tube several times to mix.

## Prepare PhiX Control Library

1 In a microcentrifuge tube, add 2 µl of stock 10 nM PhiX library to 8 µl 1X EBT buffer to yield 10 µl of 2 nM PhiX library.

2 Add 10 µl of 0.1 N NaOH to 10 µl of 2 nM PhiX library to yield 20 µl of 1 nM PhiX library.

3 Vortex the 1 nM PhiX library briefly to mix, then spin at 280 xg for 1 minute.

4 Incubate the PhiX library for 4 minutes 30 seconds at room temperature to denature. Make sure that incubation time does not exceed a maximum of 5 minutes.

5 Add 980 µl of pre-chilled HT1 to the 20 µl denatured PhiX library to make 20 pM PhiX library.

NOTE
The denatured 20 pM PhiX library can be stored up to three weeks at -15° to 25°C as single-use aliquots. After three weeks, cluster numbers tend to decrease.

## Prepare Samples for Sequencing

1 If the **LNP** plate was stored frozen, thaw at room temperature.

2 Centrifuge the **LNP** plate at 1,000 xg at 20°C for 1 minute to collect condensation.

3 Determine the samples to be pooled for sequencing.

4 If the **LNP** plate was stored frozen, using a P200 multichannel pipette set to 40 µl, mix each library to be sequenced by pipetting up and down 3–5 times. Change tips between samples.

## Library Denaturing and Pooling

When using the TruSight Tumor assay, Illumina recommends sequencing four tumor samples (8 libraries total) per run when using V2 chemistry. This process is outlined in the following steps. If sequencing a different number of samples, adjust accordingly.

NOTE
Each sample is represented by two libraries, which are generated from the TruSight Tumor Oligo Pools (FPA and FPB). It is critical that the FPA and FPB libraries for each sample be run together on the same flowcell in order for the MiSeq software to properly analyze the results for each sample.

NOTE
Control libraries generated from ACD1 with ACP1 must be pooled and run separately from those prepared with FPA and FPB, as they will require a longer MiSeq run of 151 cycles.

1. Add 10 µl of 1N NaOH to 140 µl EBT buffer. Vortex the solution.
2. Transfer 5 µl of each 4 nM library to be sequenced from the **LNP** plate to its own tube in an eight-tube PCR strip tube.

   NOTE
   After use, the sealed LNP plate may be stored at -15° to -25°C for up to 7 days.
3. Add 15 µl of the NaOH/EBT solution to each 5 µl of library and incubate for 5 minutes at room temperature.
4. Label 1 microcentrifuge tube "**PAL**" (Pooled Amplicon Library).
5. Add 10 µl of each library/NaOH/EBT solution (there should be 8) into the PAL tube.
6. Mix the pooled libraries well by pipetting up and down 10 times.
7. Label 1 microcentrifuge tube "**DAL**" (Diluted Amplicon Library).
8. In the DAL tube, mix 792 µl of HT1 with 8 µl of 20 pM PhiX library. Using the same tip, pipette up and down 3–5 times to rinse the tip and ensure complete transfer.
9. Add 8 µl from the PAL tube to the DAL tube containing HT1 and PhiX. Using the same tip, pipette up and down 3–5 times to rinse the tip and ensure complete transfer.
10. Mix DAL by vortexing the tube at top speed.

NOTE
If you would like to make and save additional DAL from the remaining 72 µl of unused PAL for future runs, it may be stored at -15° to -25°C for up to 3 days. Longer storage may lead to sub-optimal cluster densities.

11 Centrifuge the DAL tube at 1,000 xg at 20°C for 1 minute to collect contents.

12 Incubate the DAL tube in a heat block at 96°C for 2 minutes.

13 After the incubation, invert the DAL tube 1–2 times to mix. Incubate immediately in the ice-water bath for 5 minutes, then transfer contents to the template position in the MiSeq reagent cartridge.

NOTE
The heat denaturation and cooling steps must occur immediately before loading the DAL into the MiSeq reagent cartridge to ensure efficient template loading onto the flow cell.

14 Proceed to library sequencing as instructed in the *MiSeq System User Guide*.

# Supporting Information

Appendix A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# Introduction

The protocols described in this guide assume that you are familiar with the contents of this appendix, have confirmed your kit contents, and have obtained all of the requisite equipment and consumables.

# Acronyms

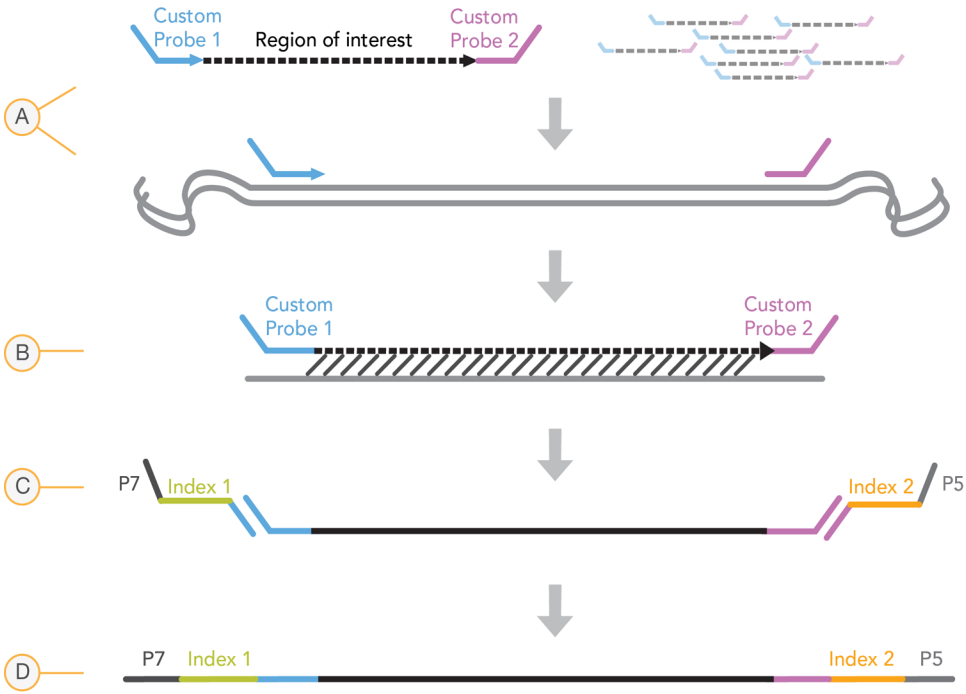
Table 2 TruSight Tumor Sample Preparation Acronyms

| Acronym | Definition |
|---|---|
| ACD1 | Amplicon Control DNA 1 |
| ACP1 | Amplicon Control Oligo Pool 1 |
| CLP | CLean-up Plate |
| DAL | Diluted Amplicon Library |
| EBT | Elution Buffer with Tris |
| ELM3 | Extension Ligation Mix 3 |
| FPA | TruSight Tumor Oligo Pool A |
| FPB | TruSight Tumor Oligo Pool B |
| FPU | Filter Plate Unit |
| HT1 | Hybridization Buffer |
| HYP | HYbridization Plate |
| IAP | Indexed Amplification Plate |
| LNP | Library Normalization Plate |
| OHS3 | Oligo Hybridization for Sequencing Reagent 3 |
| PAL | Pooled Amplicon Library |
| PMM2 | PCR Master Mix 2 |
| QCP | Quality Control Primers |
| QCT | Quality Control Template |

| Acronym | Definition |
|---|---|
| SGP | StoraGe Plate |
| SW1 | Stringent Wash 1 |
| TDP1 | TruSeq DNA Polymerase 1 |
| UB1 | Universal Buffer 1 |

# How Does the TruSight Tumor Sample Preparation Assay Work?

For each amplicon two pairs of oligos are designed. One pair is complementary to one strand and another pair to the opposite strand. In separate wells of a 96-well plate, these oligos hybridize to the genomic DNA, followed by extension and ligation to form DNA templates consisting of the regions of interest flanked by universal primer sequences. Using indexed primers supplied with the kit, DNA templates are then amplified by PCR. The library products are then pooled into a single tube, and sequenced on the MiSeq System.




**A** Hybridization of custom oligonucleotide probes
**B** Extension and ligation
**C** Addition of indices and sequencing adapters by PCR
**D** Final amplicon ready for sequencing with MiSeq

# TruSight Tumor Sample Preparation Process Overview

The TruSight Tumor Sample Preparation process overview can be summarized into the following steps:

## Place Your Order

Place your order directly through your MyIllumina account. Go to the TruSight Tumor Sample Preparation product page on the Illumina website. A MyIllumina account is required. When placing your order, make sure you also order MiSeq reagents and additional components needed to perform the TruSight Tumor Sample Preparation. See the *TruSight Tumor Sample Preparation Kit Contents* on page 42 in this guide for information on additional components such as filter plates.

## Download Your Manifest Files

Two manifest files are required for TruSight Tumor Sample Preparation sequencing on the MiSeq. They provide the list of regions targeted by the assay, which is used by the MiSeq for alignment and analysis. The manifest files can be downloaded from the TruSight Tumor Sample Preparation Support page. Go to the TruSight Tumor Sample Preparation support page and click **Downloads**.

## Prepare Libraries and Sample Sheet

Prepare your libraries using the protocol detailed in this user guide. In parallel, you must prepare a sample sheet, which will be used by the MiSeq to identify each sample and its corresponding index. To prepare your sample sheet, use the Illumina Experiment Manager, a wizard-based application which allows for the recording of your sample ID, workflow, indices, and other parameters applicable to your 96-well plate. The Illumina Experiment Manager can be run on any Windows platform. You can download the Illumina Experiment Manager from the Illumina website at http://www.illumina.com. Go to the TruSight Tumor Sample Preparation support page and click **Downloads**.

## Sequence Libraries on MiSeq

TruSight Tumor Sample Preparation libraries must be sequenced on a MiSeq sequencing system using a paired-end sequencing run. For more details on using the MiSeq instrument or setting up your run, please see the *MiSeq System User Guide*.

## Analyze Data with Illumina-Supported Software

**Analyze Data with MiSeq Reporter** — MiSeq Reporter processes the base calls generated by the MiSeq sequencing system. It is an on-instrument software, which is built in to the instrument's processes. MiSeq Reporter produces information such as alignment and structural variants. For TruSight Tumor libraries, the Amplicon - DS workflow is provided as a plug-in for MiSeq Reporter. This workflow produces aligned reads in the BAM format and outputs variants in .vcf files. These files merge the variant information obtained from sequencing of both strands. For more information about this software, please see the *MiSeq System User Guide*, *MiSeq Reporter Amplicon - DS Workflow Reference Guide*, or MiSeq Reporter's online help (http://www.illumina.com/help/miseq_reporter/default.htm).

# TruSight Tumor Sample Preparation Kit Contents

The TruSight Tumor Sample Preparation Kit contains the following components and is shipped on dry ice unless specified otherwise below. As soon as you receive your kit, store the kit components at the specified temperatures and in designated pre-amplification and post-amplification areas.

## TruSight Tumor Sample Preparation Kit (Catalog # FC-130-2001; TG* Catalog # TG-130-2001)

- Box 1, Pre-Amplification

| Acronym | Reagent Name | Storage Temperature | Area |
|---|---|---|---|
| ACD1 | Amplicon Control DNA 1 | -15° to -25°C | Pre-Amp |
| ACP1 | Amplicon Control Oligo Pool 1 | -15° to -25°C | Pre-Amp |
| OHS3 | Oligo Hybridization for Sequencing Reagent 3 | -15° to -25°C | Pre-Amp |
| ELM3 | Extension Ligation Mix 3 | -15° to -25°C | Pre-Amp |
| PMM2 | PCR Master Mix 2 | -15° to -25°C | Pre-Amp |
| TDP1 | TruSeq DNA Polymerase 1 | -15° to -25°C | Pre-Amp |
| SW1 | Stringent Wash 1 | 2° to 8°C | Pre-Amp |
| UB1 | Universal Buffer 1 | 2° to 8°C | Pre-Amp |

NOTE

*TG-labeled consumables include features intended to help reduce the frequency of revalidation. They are available only under supply agreement and require you to provide a binding forecast. Please contact your account manager for more information.

WARNING

**This set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.**

For more information, see the MSDS for this kit, at http://www.illumina.com/msds.

▸ Box 2, TruSight Tumor Oligo Set, Pre-Amplification

| Acronym | Reagent Name | Storage Temperature | Area |
|---|---|---|---|
| QCP | Quality Control Primers | -15° to -25°C | Pre-Amp |
| FPA | TruSight Tumor Oligo Pool A | -15° to -25°C | Pre-Amp |
| FPB | TruSight Tumor Oligo Pool B | -15° to -25°C | Pre-Amp |
| QCT | Quality Control Template | -15° to -25°C | Pre-Amp |

▸ Box 3, Post-Amplification

| Acronym | Reagent Name | Storage Temperature | Area |
|---|---|---|---|
| HT1 | Hybridization Buffer | -15° to -25°C | Post-Amp |
| EBT | Elution Buffer with Tris | Room temperature | Post-Amp |

## TruSight Tumor Sample Preparation Index Kit

▸ Box 1, Pre-Amplification

| Reagent Name | Storage Temperature | Area |
|---|---|---|
| i5 Index Primers, A501 to A508 (8 tubes) | -15° to -25°C | Pre-Amp |
| i7 Index Primers, A701 to A712 (12 tubes) | -15° to -25°C | Pre-Amp |

▸ Box 2, Pre-Amplification

| Reagent Name | Storage Temperature | Area |
|---|---|---|
| i5 Index Tube Caps, White | Room temperature | Pre-Amp |
| i7 Index Tube Caps, Orange | Room temperature | Pre-Amp |

## Additional Required Components

| Consumable | Catalog # | Storage Temperature | Area |
|---|---|---|---|
| TruSeq Custom Amplicon Filter Plate | FC-130-1006 | Room temperature | Pre-Amp |
| TruSeq Index Plate Fixture and Collar Kit (Reusable) | FC-130-1007 | Room temperature | Pre-Amp |

# User-Supplied Consumables

| Quantity | Consumable | Supplier |
|---|---|---|
| As needed | PhiX control kit | Illumina, Part # 15034071 |
| As needed | 10 N NaOH (prepare from tablets or use a standard solution) | General lab supplier |
| As needed | 100x TE Buffer Sigma-Aldrich Part # T9285 (Dilute to 1x TE Buffer) | General lab supplier |
| 3 | 96-well skirted PCR plates, 0.2 ml, polypropylene | Bio-Rad, Part # MSP-9601 |
| 3 | 96-well storage plates, 0.8 ml (MIDI plates) | Fisher Scientific, Part # AB-0859<br>Fisher Scientific, Part # AB-0765 |
| As needed | Agencourt AMPure XP, 60 ml kit | Beckman Coulter, Part # A63881/A63880 |
| 3 | Adhesive aluminum foil seal | Beckman Coulter, Part # 538619 |
| As needed | Conical tubes, 15 ml | General lab supplier |
| 2 | Eppendorf microcentrifuge tubes (screw top recommended) | General lab supplier |
| 40 ml | Ethanol, 200 proof for molecular biology | General lab supplier |
| As needed | Microseal 'B' adhesive seals | Bio-Rad, Part # MSB-1001 |
| 2 | PCR Eight-Tube Strips | General lab supplier |
| As needed | Solution basin, PVC, non-sterile (trough) | Labcor, Part# 730-001 |
| As needed | Agarose gel (2% or 4%) | General Lab Supplier |
| As needed | DNA 1000 Kit for Bioanalyzer | Agilent 5067-1504<br>(for 300 samples) |
| As needed | DNA molecular weight markers | General Lab Supplier |
| As needed | Ice bucket | General Lab Supplier |

# Equipment

## Pre-PCR

| Equipment | Supplier |
|---|---|
| 37° Incubator | Forced Air Oven, VWR International or comparable |
| Heat Block, 96-well | Scigene, Hybex Microsample Incubator for PCR plate<br>Note: This model is recommended for this assay. Passive cooling, as opposed to active cooling performed in a PCR thermocycler, is recommended for maximum target enrichment specificity and uniformity. |
| Tabletop Centrifuge | General lab supplier (Plate centrifuge that attains designated speeds of protocol) |

NOTE
Use a dedicated set of pipettes, pipette tips, vortexer, and centrifuge for pre-amplification steps.

## Post-PCR

| Equipment | Supplier |
|---|---|
| Magnetic Stand-96 | Invitrogen DynaMag™-96 Side Skirted |
| Post-PCR Plate Shaker | Q Instruments, BioShake iQ High Speed Thermal mixer, Part#1808-0506 or Q Instruments, BioShake XP High Speed mixer, Part#1808-0505 |
| Tabletop Centrifuge | General lab supplier (Plate centrifuge that attains designated speeds of protocol) |
| Gel Electrophoresis Supplies and Apparatus | General lab supplier |
| Bioanalyzer System | Agilent Technologies |
| Heat Block for 1.5 ml centrifuge tubes | General lab supplier |

NOTE
Use a dedicated set of pipettes, pipette tips, vortexer, heat block, and centrifuge for post-amplification steps.

## Thermal Cycler

The following table lists the recommended settings for selected thermal cycler models. Illumina recommends that you validate any thermal cyclers not listed below if your lab has not yet performed the TruSight Tumor Sample Preparation protocol.

| Thermal Cycler | Temp Mode | Lid Temp | Vessel Type |
|---|---|---|---|
| Bio-Rad DNA Engine Tetrad 2 | Calculated | Heated, Constant at 100°C | Polypropylene plates and tubes |
| MJ Research DNA Engine Tetrad | Calculated | Heated | Plate |
| Eppendorf Mastercycler Pro S | Gradient S, Simulated Tube | Heated | Plate |

NOTE

The gDNA qPCR evaluation was optimized on the Illumina Eco Real-Time PCR System and the Bio-Rad CFX396 System. If using other machines, the protocol should be verified prior to use.

# Prevent PCR Product Contamination

The PCR process is commonly used in the laboratory to amplify specific DNA sequences. Unless proper laboratory hygiene is used, PCR products can contaminate reagents, instrumentation, and genomic DNA samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay normal operations.

Make sure that the lab is set up appropriately to reduce the risk of PCR product contamination:

- **Physically Separate Pre-PCR and Post-PCR Areas**
  - Physically separate laboratory space where pre-PCR processes are performed (DNA extraction, quantification, and normalization) from the laboratory space where PCR products are made and processed (post-PCR processes).
  - Never use the same sink to wash pre-PCR and post-PCR troughs.
  - Never share the same water purification system for pre-PCR and post-PCR processes.
  - Store all supplies used in the protocols in the pre-PCR area, and transfer to the post-PCR area as needed.
- **Use Dedicated Equipment and Supplies**
  - Dedicate separate full sets of equipment and supplies (pipettes, centrifuges, oven, heat block, etc.) to pre-PCR and post-PCR lab processes, and never share between processes.
  - Dedicate separate storage areas (freezers and refrigerators) to pre-PCR and post-PCR consumables.

Because the pre- and post-amplification reagents are shipped together, it is important to unpack the reagents in the pre-PCR lab area, and then move the post-amplification reagents to the proper post-PCR storage area.

## Pre-PCR and Post-PCR Lab Procedures

To prevent PCR product contamination, it is important to establish lab procedures and follow best practices. Illumina recommends daily and weekly cleaning of lab areas using 0.5% Sodium Hypochlorite (10% Bleach).

CAUTION

To prevent sample or reagent degradation, make sure that all vapors from the cleaning solution have fully dissipated before beginning any processes.

## Daily Cleaning of Pre-PCR Area

A daily cleaning of the pre-PCR area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps to eliminate PCR product that has entered the pre-PCR area.

Identify pre-PCR areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution before beginning any pre-PCR processes. High-risk areas might include, but are not limited to, the following items:

- Bench tops
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards

## Daily Cleaning of Post-PCR Area

Reducing the amount of PCR product in the post-PCR area helps reduce the risk of contamination in the pre-PCR area. Daily cleaning of the post-PCR area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps achieve this.

Identify post-PCR areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution daily. High-risk areas might include, but are not limited to, the following items:

- Thermal cyclers
- Bench space used to process amplified DNA
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards

## Weekly Cleaning of All Lab Areas

Once a week, perform a thorough cleaning of the pre-PCR and post-PCR areas using 0.5% Sodium Hypochlorite (10% Bleach).

- Clean all bench tops and laboratory surfaces.
- Clean all instruments that are not cleaned daily.
- Thoroughly mop lab floors.
- Make sure that personnel responsible for weekly cleaning are properly trained on prevention of PCR product contamination.

### Items Fallen to the Floor

The floor is contaminated with PCR product transferred on the shoes of individuals coming from the post-PCR area; therefore, anything falling to the floor must be treated as contaminated.

- Disposable items that have fallen to the floor, such as empty tubes, pipette tips, gloves, lab coat hangers, must be discarded.
- Non-disposable items that have fallen to the floor, such as a pipette or an important sample container, must be immediately and thoroughly cleaned with a 0.5% Sodium Hypochlorite (10% Bleach) solution to remove PCR product contamination.
- Clean any lab surface that has come in contact with the contaminated item. Individuals handling anything that has fallen to the floor, disposable or non-disposable, must discard their lab gloves and put on a new pair.

# MiSeq Sample Sheet Preparation

Create your Sample Sheet for MiSeq sequencing using the Illumina Experiment Manager. Illumina recommends the Illumina Experiment Manager to prepare your Sample Plate and Sample Sheet. Select MiSeq as your instrument, then select Targeted Resequencing and Amplicon - DS as the workflow. Ensure you select the Homo Sapiens genome folder.

NOTE
The assay control prepared with ACD1/ACP1 should be given the Sample ID and Sample Name "Control" in your Sample Plate and Sample Sheet files.

| i7 Index PCR Primer | Index Sequence |
|---|---|
| A701 | ATCACGAC |
| A702 | ACAGTGGT |
| A703 | CAGATCCA |
| A704 | ACAAACGG |
| A705 | ACCCAGCA |
| A706 | AACCCCTC |
| A707 | CCCAACCT |
| A708 | CACCACAC |
| A709 | GAAACCCA |
| A710 | TGTGACCA |
| A711 | AGGGTCAA |
| A712 | AGGAGTGG |

| i5 Index PCR Primer | Index Sequence |
|---|---|
| A501 | TGAACCTT |

| i5 Index PCR Primer | Index Sequence |
|---|---|
| A502 | TGCTAAGT |
| A503 | TGTTCTCT |
| A504 | TAAGACAC |
| A505 | CTAATCGA |
| A506 | CTAGAACA |
| A507 | TAAGTTCC |
| A508 | TAGACCTA |

# Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 3 Illumina General Contact Information

| | |
|---|---|
| **Illumina Website** | www.illumina.com |
| **Email** | techsupport@illumina.com |

Table 4 Illumina Customer Support Telephone Numbers

| Region | Contact Number | Region | Contact Number |
|---|---|---|---|
| North America | 1.800.809.4566 | Italy | 800.874909 |
| Austria | 0800.296575 | Netherlands | 0800.0223859 |
| Belgium | 0800.81102 | Norway | 800.16836 |
| Denmark | 80882346 | Spain | 900.812168 |
| Finland | 0800.918363 | Sweden | 020790181 |
| France | 0800.911850 | Switzerland | 0800.563118 |
| Germany | 0800.180.8994 | United Kingdom | 0800.917.0041 |
| Ireland | 1.800.812949 | Other countries | +44.1799.534000 |

## MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at www.illumina.com/msds.

## Product Documentation

Product documentation in PDF is available for download from the Illumina website. Go to www.illumina.com/support, select a product, then click **Documentation & Literature**.

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